The role of semaphorins and their receptors in vascular development and cancer

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Summary

Semaphorins (Semas) are a large family of traditional axon guidance molecules. Through interactions with their receptors, Plexins and Neuropilins, Semas play critical roles in a continuously growing list of diverse biological systems. In this review, we focus on their function in regulating vascular development. In addition, over the past few years a number of findings have shown the crucial role that Semas and their receptors play in the regulation of cancer progression and tumour angiogenesis. In particular, Semas control tumour progression by directly influencing the behavior of cancer cells or, indirectly, by modulating angiogenesis and the function of other cell types in the tumor microenvironment (i.e., inflammatory cells and fibroblasts). Some Semas can activate or inhibit tumor progression and angiogenesis, while others may have the opposite effect depending on specific post-translational modifications. Here we will also discuss the diverse biological effects of Semas and their receptor complexes on cancer progression as well as their impact on the tumor microenvironment.

Semaphorins and their receptors:

The Semas are a large family of secreted and membrane-bound proteins originally identified as axon guidance cues and later shown to be key regulators in many biological processes including vascular development and cancer. Based on structural similarities, the Semas are grouped into 8 classes, all of which carry a N-terminal Sema domain. Semas signal via two major receptor families, Plexins and Neuropilins (Nrps or Npns). Membrane-bound Semas bind and signal directly through Plexins whereas most of the class 3 secreted Semas (Sema3A-3G) are known to bind to a holoreceptor complex consisting of Nrps as the ligand binding subunit and Plexins as the signal transducing subunit [1-3]. The exception to this rule is Sema3E, which binds its receptor Plexin-D1 directly and independently of the Nrps [4]. There are four groups of Plexins (A, B, C, and D) that have been identified so far, and similar to Semas, all Plexins have Sema domains in their extracellular region. In addition, Plexins have Met-related sequences (MRS) and glycine/proline-rich motifs in the extracellular region. The intracellular domains of Plexins do not have obvious enzymatic activity, but most of them have weak sequence similarity to GTPase activating proteins (GAPs) and exhibit GAP activity towards the small GTPase R-Ras [5]. Moreover, their intracellular regions all contain two highly conserved intracellular domains known together as the SEX-plexin domain. Recent structural work has revealed that binding of each homodimer arrangement of Sema and Plexin forms a heterodimer complex that then elicits a conformational change in the complex. This structural alteration transmits signals to the intracellular domain of Plexins [6]. In contrast to Plexins, Nrps (Nrp1 and Nrp2) have a very short cytoplasmic domain with a PDZ-binding motif at the C-terminal. The extracellular domains of Nrps contain two complement-binding domains (a1 and a2), two coagulation factor V/VIII homology domains (b1 and b2), and a MAM domain (c). Importantly, besides binding to Sema3s, Nrps also bind to a structurally different family of ligands, the VEGF family, and serve as their co-receptors [7]. Although the a1 domain is only required for Sema3 binding, the b1 domain is required for both Sema3 and VEGF binding [8]. Thus, determining the precise contributions of Sema3s and Nrps is complex because Nrps also control VEGF receptor signaling.

Semaphorins in developmental angiogenesis

It was initially suggested that Sema3s compete with VEGF to bind to Nrp1 and therefore Sema3s inhibit VEGF-induced angiogenesis. Some *in vitro* data also suggest that Sema3s negatively regulate endothelial migration, for example Sema3A inhibits endothelial cell migration and survival [9, 10]. However, *in vivo* studies using knockout mice thus far show very little evidence for the role of Sema3s in developmental angiogenesis except in the case of Sema3E (see below). In the case of Sema3A, no obvious developmental vascular patterning defect were found in mice selectively lacking Sema3-Nrp1 signaling [11] or mice lacking Sema3A [12]. In addition, Sema3B-, Sema3F-, and Sema3G-null mice are also viable and fertile with no overt vascular defects. Sema3C null mice die perinatally from cardiac defect [13]. Therefore, most of the Sema3s are perhaps not required for the early stages of developmental angiogenesis, but rather play a role during vessel remodeling and pathological angiogenesis (see other sections of the review). For example, Sema3A is expressed in endothelial cells and in an autocrine manner regulates endothelial cell migration and vessel remodeling by inhibiting integrin function [14]. However, the molecular mechanisms by which Sema3A regulates integrins are not fully understood. Moreover, recent work demonstrated that genetic deletion of Sema3A in mice results in severe renal vascular patterning defects, further support the role of Sema3A in angiogenic remodeling [15] Most Sema3s have an inhibitory function on *in vitro* endothelial cell migration assays, but whether this inhibitory effect is due to competitive Nrp binding and therefore the inhibition of VEGF signaling as was originally proposed is still unclear (see more discussion in the Nrp section).

The most obvious Sema3 member to play a key role in regulating developmental angiogenesis is Sema3E and its receptor Plexin-D1. Unlike other class 3 secreted Semas that require Nrps as an obligatory ligand-binding subunit in the Plexin/Nrp holoreceptor, Sema3E can bind and signal through Plexin-D1 independent of Nrps. As a novel ligand-receptor pair, Sema3E-Plexin-D1, plays a critical role in shaping the vascular network during development [4]. During intersomitic vessel formation, Sema3E is expressed in the caudal region of each developing somite, whereas Plexin-D1 is expressed in the intersomitic blood vessels adjacent to the somite boundary in the rostral region of each somite. Sema3E acts as a repulsive cue to restrict vessel

growth and branching in the intersomitic space. As a result, both Sema3E and Plexin-D1 knockout mice exhibit severe intersomitic vessel patterning defects. Specifically, the intersomitic vessels are no longer excluded from the normal Sema3E-expressing caudal region of the somite and extended ectopically throughout the somites, resulting in exuberant growth and a loss of the normal segmented pattern [4, 16]. Plexin-D1 morphant zebrafish exhibit similar defects in intersomitic vessel patterning [17]. In addition to its function in intersomitic vessel patterning, recent work also demonstrates that Sema3E plays a role in the initial formation of the dorsal aorta. Sema3E secreted from the notochord and lateral plate mesoderm are required for the formation of avascular regions that coordinate to sculpt the mouse dorsal aorta. In Sema3E knockout embryos, a branched aortic plexus develops abnormally with a markedly narrowed avascular midline [18]. Therefore, the repulsive gradient generated by Sema3E in the mouse somite as well as the notochord and lateral plate mesoderm determines the proper patterning of Plexin-D1-expressing intersomitic vessels and the dorsal aorta, respectively.

The molecular mechanism underlying Sema3E-Plexin-D1 signaling in vascular patterning has been linked to the well known VEGF pathway by several studies in both mouse and zebrafish [19] [20] [21] [22]. In the developing retinal vasculature, Sema3E is expressed uniformly by retinal ganglion cells (RGCs), but Plexin-D1 is selectively expressed in endothelial cells at the front of actively sprouting blood vessels and VEGF is necessary and sufficient for the dynamic expression of Plexin-D1 [19] [20]. In turn, Sema3E-Plexin-D1 signaling then negatively regulates VEGF-induced Dll4-Notch signaling and subsequently changes the tip/stalk cell ratio. As a result, a lack of Sema3E or Plexin-D1 in mice leads to a disruption of vascular patterning in retinal vasculature, generating an uneven growing front and a less-branched network [19]. It has been suggested that VEGF-Notch feedback and dynamic tip and stalk cell position-shuffling promotes reiterative sprouting and branching and thus robust network formation during angiogenesis [23]. Because Sema3E-Plexin-D1 signaling negatively regulates VEGF signaling, it will be interesting to know whether Sema3E-Plexin-D1 is actively involved in regulating the timing of the tip/stalk cell switching during angiogenesis.

In zebrafish, the cross-talk between VEGF and Sema3E-Plexin-D1 signaling are found to be mediated by soluble Flt1 (sFlt1). Plexin-D1 antagonizes VEGF signaling by promoting the expression of sFlt1, a Flk1/VEGFR2-decoy, in endothelial cells, and the loss of Plexin-D1 induces the sprouting of ectopic segmental arteries [17] [21]. Therefore, the interplay between Plexin-D1 and VEGFR seems to be evolutionarily conserved during vascular development. It will be interesting to determine whether Plexin-D1 functions to regulate VEGFR signaling via enhancing sFlt1 expression in mammalian vascular development. In addition, since the elimination of Sema3E in zebrafish does not lead to the phenotype seen in mammals [24], it will be interesting to identify the ligand(s) associate with Plexin-D1 in fish. This result clearly demonstrates the existence of both evolutional similarity and difference across species.

Finally, cross-talk between Sema3E-Plexin-D1 and VEGF has also been shown during pathological angiogenesis, which is achieved through the activation of the small GTPase RhoJ. In contrast to the VEGF-induced activation of Cdc42, which plays a crucial role in filopodia protrusion, Sema3E-induced activation of RhoJ mediates endothelial filopodia retraction[20].

It should be mentioned that like in the nervous system, Semas can also function as an attractant in some cases. Although so far most of the data show that Semas inhibit endothelial cell migration, some Semas also exhibit pro-angiogenesis function. For example, Sema4D-Plexin-B1 signaling promotes endothelial cell migration and tube formation [25, 26]. Moreover, Sema3C has been shown to increase endothelial migration and proliferation in renal glomeruli [27].

Neuropilins in developmental angiogenesis

The role of Nrps in angiogenesis is complicated by the fact that they bind to both Sema3s and VEGF families. Large amounts of *in vitro* work trying to elucidate the precise function of Nrps in angiogenesis

have generated controversial results and the interpretations of these studies make Nrps even more mysterious. For example, when Nrp1 was originally identified as a receptor for VEGF, the authors showed that in heterologous cells, Nrp1 and VEGFR2 were capable of forming a complex that could be immunoprecipitated only in the presence of VEGF-165, which binds both receptors. Therefore, they proposed that VEGF forms a bridge between the two proteins [28]. It has been shown that when stimulated with VEGF, endothelial cells only expressing Nrp1 do not migrate, endothelial cells only expressing VEGFR2 migrate, whereas those expressing both Nrp1 and VEGFR2 showed enhanced chemotaxis compared with endothelial cells expressing VEGFR2 alone [10] [29]. Nrp1/VEGFR2 complex formation has been shown to require the PDZ-binding domain of Npn-1 [30]. A recent *in vitro* loss of function study showed that when VEGF binding to Npn1 was abolished, the Nrp1/VEGFR2 association was disrupted [31]. However, it has not been shown definitively whether Nrp1 in association with VEGFR2 has a higher affinity for VEGF than VEGFR2 alone. It has also not been demonstrated whether Nrp1 increases the intrinsic catalytic activity of the VEGFR2 kinase domain. Moreover, there is disagreement in the literature as to whether the association of Nrp1 and VEGFR2 is VEGF-dependent, with some groups reporting VEGF-induced Nrp1 association with VEGFR2 [28, 32], while other studies were able to co-immunoprecipitate VEGFR2 and Nrp1 in the absence of VEGF [33] [34]. These disagreements in literature could be due to different *in vitro* settings and overexpression systems. *In vivo* loss of function experiments are required to address the precise role of Nrps in developmental angiogenesis.

Some *in vitro* studies have also proposed that VEGF binding to Nrp1 can lead to signaling through Nrp1 itself. For example, one study used a chimeric receptor consisting of the extracellular portion of the EGF receptor and the intracellular domain of Nrp1 and found that activation of this receptor with EGF was able to induce cell migration that was not present when cells were transfected with wild-type Nrp1 or wild-type EGFR [35]. Although Nrp1 has a very short intracellular domain (ICD) with little homology to known signaling domains, the very C-terminal of the ICD contains a PDZ-binding motif. A yeast two-hybrid screen was used to screen for proteins that interact with the ICD, and a protein called GIPC was identified and

shown to bind to the PDZ binding motif [36]. However, GIPC knockout mice have a fairly mild vascular phenotype compared to the Nrp1 null [37]. A separate study found that VEGF-induced migration of HUVECs was reduced in cells that were infected with an adenovirus vector encoding Nrp1 lacking its C-terminus [38]. Yet, mice lacking the Nrp1 ICD domain were shown to have none of the embryonic vascular or cardiac defects of Nrp1 nulls [39].

Several mouse models have been generated that allow us to delineate Nrp1 function in angiogenesis *in vivo*. First, consistent with its dual binding to Sema3 and VEGF, Npn-1 null mice die very early during embryogenesis and exhibit both neural and vascular defects [40, 41]. Second, mice lacking Nrp1 specifically in endothelial cells demonstrated that Nrp1 is cell autonomously required for developmental angiogenesis in endothelial cells [11]. When the conditional Nrp mice were generated and crossed with an endothelial-specific Cre line (*Tie2-Cre*), the mutant embryos exhibit severe vascular defects including large and abnormal clumps of vascular aggregates. In contrast, neuronal-Cre-driven Nrp1 mutants ($Npn1^{flox/flox}$; *Nestin-Cre*) have none of the vascular defects of the endothelial-Cre-driven mutants, but do recapitulate the defasciculation defects seen in the Nrp1 null. Therefore, Nrp protein is absolutely essential in endothelial cells for developmental angiogenesis. Third, to further address which ligands are responsible for Nrp1's anti-angiogenesis function in endothelial cells, another mouse line, $Npn1^{Sema-}$ mice carrying a mutation that specifically abolishes Sema3 binding but not VEGF binding were generated [11]. Like the neuronal-specific knockout, the $Npn1^{Sema-}$ mice have a variety of neural developmental defects, but surprisingly, did not exhibit any vascular phenotypes. This *in vivo* result demonstrated that Sema3A-Npn1 interaction is critical for nervous system development, while also indicating that this interaction is dispensable for vascular development. Therefore, the severe vascular defects observed in mice lacking Npn-1 in endothelial cells (*Tie-2 Cre;* $Npn\text{-}1^{fl/fl}$) together with the absence of vascular defects in mice selectively abolishing Sema3-Npn1 binding ($Npn1^{Sema-}$) suggest that it is most likely that VEGF-Npn-1 interaction that is responsible for the critical function of Npn-1 in developmental angiogenesis. Mice carrying a mutation that specifically

abolishes VEGF binding without affecting Sema3 binding (*Npn-1* $^{VEGF-}$) will be needed to test this hypothesis.

Although *in vitro* and *in vivo* studies over the past decades have furthered our understanding of the role of Nrp1 in angiogenesis, more questions have also been raised. So far there still remains several possible mechanisms underlying Nrp function in endothelial cells. One possibility is that the VEGF-Nrp1 interaction is critical for angiogenesis because it enhances VEGFR2 signaling. Another hypothesis raised recently is that Npn1 aids in VEGFR2 recycling following VEGF stimulation. In heterologous cells expressing only VEGFR2, VEGF stimulation leads to the internalization of VEGFR2 into Rab7-positive vesicles that are destined for degradation [42]. However, in the presence of Nrp1, VEGF stimulation leads to internalization of the Nrp1/VEGFR2 complex into Rab11-positive vesicles, which are then recycled back to the cell surface. Whether this occurs *in vivo* is still awaiting examination. Finally, since Nrp1 has a large extracellular domain, it is formally possible that a ligand other than Sema3 and VEGF mediates its function in developmental angiogenesis. For example, when Nrp was initially identified as an adhesion molecule, it was suggested that a "ligand" may exist to mediate its function. However, such ligand has not yet been identified.

Plexins in developmental angiogenesis

The function of plexins in developmental angiogenesis is relatively unknown in comparison to Semas and Nrps. First, it is unclear whether plexins are actually expressed in endothelial cells, although several studies have suggested weak endothelial cell expression of PlexinA [14]. PlexinA1- PlexinA2-, PlexinA-3 and PlexinA4-null mice are all viable and fertile with no vascular abnormalities reported. Thus far, Plexin-D1 is the best characterized plexin that shown strong and broad endothelial cell expression and *in vivo* functional requirement for developmental angiogenesis. Please see details above in the role of Sema3E section, and also another recent review [43].

Semas control tumour progression

Sema3s have a direct effect on cancer cells. The first evidence suggesting a potential role of Semas in controlling tumor angiogenesis derived from the identification of Sema3F as tumor suppressor in distinct deletion of 3p21.3 chromosomal region in lung cancer [44, 45]. Stemming from these data, several findings in recent years described Sema3s as modulators of cancer growth. For instance Sema3F inhibit cancer growth and metastasis formation in different xenograft mouse models [46]. In addition, it has been observed that the transcription repressor zinc finger E-box binding homeobox-box (ZEB)-1, highly up-regulated in lung cancer cells, inhibits the expression of Sema3F in these cells [47] and that, the up-regulation of Id2 in metastatic tumors over-expressing c-myc, inhibits Sema3F expression [48]. Other Sema3s, such as Sema3A, Sema 3B and Sema3F have anti-tumor effects. Indeed, Sema3A blocks breast cancer cell migration and invasion through an inhibitory autocrine mechanism involving its receptor Nrp-1 that modulates tumour cell motility [49, 50]. Moreover, the over-expression of Sema3A halts the migration and invasion in prostate cancer cells [51] and strongly impairs cell migration, invasion and suppress tumor growth and metastasis in melanoma cells [52]. Sema3B, similarly to Sema3F was identified as potential tumor suppressor. Sema3B is lost in lung cancer, and in neuroblastoma [53, 54] and can act directly on lung tumor cells by inhibiting anchorage-independent growth and inducing apoptosis [53].

Several reports described that Semas other than Sema3s regulate tumor progression. For instance Sema4D, a single pass transmembrane protein, binding plexin-B1 receptor in tumor cells and by inducing the phosphorylation of Met and Ron, promotes tumor invasion and tumor metastasis [55, 56]. In addition, Sema4D induces tumor progression of head and neck squamous cell carcinomas [57]. Another example is Sema6D, a membrane-anchored semaphorin that use Plexin-A1 as its receptor. Interestingly, Sema6D, binding Plexin-A1 in complex with VEGFR-2, mediates survival and anchorage-independent growth of malignant mesothelioma cells [58]. Since both Plexin-A1 and VEGFR-2 are expressed on endothelial cells (ECs) it is possible that Sema6D could act also as a pro-angiogenic factor, but, this specific effect on the vasculature, has to be clarified.

Semas regulate tumor angiogenesis

A growing body of evidence so far implicate Semas as important modulators of both physiological and tumor angiogenesis [59-61] [62]. Several *in vitro* and *in vivo* studies demonstrate that Sema3s regulate angiogenesis. For instance Sema3A synergize with Sema3F to induce endothelial cell apoptosis [9] Interestingly, Sema3A and Sema3F, are expressed by ECs and may exert their effects in an autocrine fashion. Indeed, endogenous Sema3A controls, through autocrine loops, EC motility and vessel remodeling by inhibiting integrin function [63].

Sema3s controls tumor angiogenesis as well. In fact, it has been recently demonstrated, in different mouse models of spontaneous tumorigenesis, i.e. RIP-Tag2 neuroendocrine pancreatic tumour, K14-HPV16 skin carcinoma and HPV16/E_2 uterine cervix carcinoma that Sema3A is an endogenous angiogenic inhibitor that is lost during tumor progression, when abnormal angiogenesis takes place. Notably, its re-expression in RIP-Tag2 tumors by adeno-associated virus (AAV)-8 inhibits tumour growth, decreases vascular density, enhances pericyte coverage, and significantly enhances the survival of treated animals compared with controls [64]. Other studies, describing that systemic delivery of Sema3A in different mouse models of cancer impairs angiogenesis and metastasis formation [65], further highlights the anti-angiogenic and anti-tumor effect of Sema3A. Re-expression of Sema3A in the tumors results in vessel normalization and in reduced tumor hypoxia. These findings further corroborate several evidence describing that the poorly functional tumor vasculature is due to an imbalance between pro- and anti-angiogenic factors as result of an overproduction of pro-angiogenic factors, such as VEGF [66] and a loss of angiogenic inhibitors such as Sema3A (and Sema3F) [64]. The restoration of this equilibrium in tumors induces a more functional and normalized vasculature [67]. It is well documented that tumor vessel normalization, a process occurring in response to the anti-angiogenic therapies that renders the tumor vasculature more efficient in delivering oxygen and drugs, represent a remarkably advantageous anti-cancer strategy, being also able to favor chemotherapy delivery and response to radiotherapy [66, 68, 69]. In this context, more recent data describe that Sema3A, by normalizing the vasculature, overcomes the resistance to the anti-angiogenic therapies. In fact, the simultaneous administration of Sema3A with the tyrosine kinase (TK) inhibitor Sunitinib or with the anti-VEGFR-2 antibody DC101, by increasing the normalization window and inhibiting tumor hypoxia, efficiently halts metastasis formation induced by these drugs in both RIP-Tag2 and HPV16/E_2 mouse models

[70]. In addition, the administration of Sema3A as single agent or combined with Sunitinib in RIP-Tag2 insulinoma, by improving vessel function, enhances the amount of doxorubicin delivered to the tumors. Interestingly, Sema3A-mediated reduction of tumor hypoxia, is able to lessen several hypoxia-induced pathways [70], such as hypoxia-inducible factor (HIF)-1α and NF-*k*B transcription factor, to inhibit Met TK receptor and to halt the epithelial-mesenchymal transition (EMT), shown to be highly involved in the promotion of a pro-invasive and metastatic phenotype [71, 72]. The underlying mechanisms regulating the pro-normalizing effects of Sema3s in cancer remain unclear. For instance, it remains to be elucidated how Sema3A (or other Sema3s) modulate the recruitment of resident or bone-marrow-derived perivascular cells to tumor vessels and which receptors, such as Nrps and PlexinAs expressed in both ECs and pericytes and their signaling pathways, are involved in this process.

Another important Sema3, first described to have a potent direct anti-angiogenic activity, is Sema3F. In fact Sema3F blocks tumor angiogenesis and metastasis formation in mouse models of cancer [46, 73]. Very recently it has been shown that, similarly to Sema3A, Sema3F may act as an vascular normalizing agent. Indeed, it has been observed the down-modulation of Sema3F in Schwannomas in which the expression of the onco-suppressor merlin/ neurofibromatosis-2 is lost, releases the pro-angiogenic activity of VEGF-A, that, in turn, induces tumor vessel abnormalization [74]. Of note, re-expression of Sema3F in merlin/ neurofibromatosis-2 null cancer cells efficiently decreased tumor growth and normalizes the vasculature by reducing vessel area and permeability and by increasing pericyte vessel coverage [75]. Also Sema3E, similarly to Sema3A and Sema3F inhibits tumor angiogenesis. In fact Sema3E exert its anti-angiogenic effect through PlexinD1 in endothelial cells [76]. Differently from Sema3A and Sema3F, the furin-dependent proteolitic cleavage of Sema3E generates a 61 kDa isoform capable to enhance EC migration and promote metastatic spreading of breast cancer cells [77]. Moreover, by binding to PlexinD1, the 61 kDa Sema3E protein induces PlexinD1 association to the ErbB2 receptor and the consequent activation the ErbB2/neu oncogenic kinase in tumor cells [78]. Notably, recent studies shows that a furin non-cleavable form of Sema3E that binds to PlexinD1 but does not induce the association with ErbB2, strongly inhibits angiogenesis and hampers metastatic spreading in different mouse models of cancer [79]. Other Sema3s, besides their direct tumor properties, display anti-angiogenic effects. For instance, studies demonstrate that Sema3B is an efficient angiogenic inhibitor in experimental angiogenesis, and that this inhibitory activity is

abrogated by furin-like pro-protein convertases [80]. More recently, a new role has been described for Sema3D in hampering tumor angiogenesis in glioblastoma tumor mouse model [81].

Probably, the most studied pro-angiogenic Sema is Sema4D. The soluble cleaved extracellular portion of Sema4D, promotes tumor angiogenesis by binding PlexinB1 [25, 57]. Interestingly, the binding of Sema4D to Plexin-B1, enhances Met phosphorylation inducing, in turn, tumor angiogenesis, invasion and metastasis dissemination [26]. Another potent pro-angiogenic Sema is Sema5A, a membrane-anchored Sema that, binding to PlxnB3, strongly enhance angiogenesis and metastasis formation in tumor pancreatic mouse models [82].

Semas modulate the function of tumor-associated macrophages and fibroblasts

A growing body of evidences described how tumour-associated inflammatory cells (e.g. macrophages and neutrophils) and cancer-associated fibroblasts (CAFs), represent key players in the regulation of cancer angiogenesis, invasion and metastatic dissemination [83, 84]. Studies have recently demonstrated that Semas and their receptors can regulate tumor angiogenesis and progression acting on these stroma cell types.

Sema and inflammation in cancer

Tumour angiogenesis is modulated by the recruitment of a series of bone marrow-derived cells (BMDCs) [83]. Among these cells, tumour-associated macrophages (TAMs) promote tumour angiogenesis [85] as well as the acquired resistance to anti-VEGF-A/VEGFR therapies [86]. Even though recent data demonstrate the presence in cancers of several subtypes of BMDCs with different and specific properties [83, 85], it conceivable to generalize that the shift of TAMs from an anti-tumor M1 polarization to a M2 alternatively activated phenotype induces tumor angiogenesis and support tumor progression [87].

It is known that Semas regulate the immune system [88] and modulate the function of TAMs, contributing to the regulation of tumor angiogenesis. For instance, Sema3B, considered potential tumor suppressor and down-modulated in many cancers [53, 54], surprisingly, can promote metastasis formation by recruiting TAMs to the tumors. Indeed, Sema3B induces the production of IL-8 in cancer cells that contribute to the recruitment of TAMs that sustain tumor angiogenesis and progression [89]. Also Sema4A, a membrane-anchored Sema that binds to plexinD1, has a dual effect on angiogenesis. Indeed, it has been shown that

Sema4A impairs physiological angiogenesis by acting directly on endothelial cells [90]. Recent studies demonstrate that Sema4A, binding Plexin-D1 expressed in macrophages, exerts a pro-angiogenic effect by enhancing VEGF-A production in these cells [91]. Notably, Sema4A is greatly expressed in macrophages activated by inflammatory stimuli and recruited at the injured area in a cardiac ischemia/reperfusion model [91], suggesting its potential role in regulating pathological angiogenesis. Interestingly, Sema7A, shown to promote experimental angiogenesis [92], also enhances the production of several pro-inflammatory molecules (e.g., IL-8, Il-6 and IL-1β) in macrophages and increases theirs migration [93]. Sema4D secreted by TAMs enhances tumor angiogenesis and contributes to the maturation of tumor blood vessels. Interestingly, a reduced recruitment to the tumors have been observed for TAMs derived from Sema4D knockout mice, compared with those derived from control animals [94].

Recent findings show that certain Semas or Nrp-1 or other Sema receptors may define specific BMDC populations that can be recruited into cancers. For instance different Semas and their receptors (such as Nrp-1, PlxnA1 and Sema6D) are specifically expressed by subsets of myeloid cells in cancers [95] and Nrps and PlxnAs are differently modulated in M2- compared to M1-polarized human macrophages [96]. It has been shown that changes in TAMs polarization can regulate tumor vessel normalization. In fact, recent data demonstrate that, in different mouse models of cancer, the histidine-rich glycoprotein (HRG) hampers cancer metastasis and normalizes the tumor vasculature by switching M2-like into M1-like phenotype. Notably, both Sema3A and VEGF-A are able to recruit a Nrp-1-expressing BMDCs that promote arterial maturation during physiological angiogenesis [97]. More recent studies shows that Sema3A recruits a specific subset of Nrp-1^{+}, Cd11b^{+}, Gr-1^{-}, Tie-2^{-} monocytes (NEM) in tumors mouse models of cancer capable to block cancer growth and to normalize the vasculature [98]. In addition, this specific NEM sub-population, can be purified from BM of normal mice and retains its anti-tumor and pro-normalizing effects when injected in tumor-bearing mice [98]. Interestingly, while Sema3A exerts a chemoattractant effect on NEMs, it induces apoptosis in M2 human macrophages, known to express Nrp-1 [96]. These findings indicate that Semas and, in particular, Sema3s may differentially regulate macrophages depending on the expression of specific Sema3 receptors in myeloid populations present in cancers, and, consequently, regulate tumour angiogenesis.

Sema and cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) or myofibroblasts, derived from mesenchymal stem cells, BM-derived or local fibroblasts, promote tumor angiogenesis and cancer progression [83, 84, 99]. To date there are no direct experimental evidences describing effects of Semas on CAFs, but recent data suggest that Sema3 receptors may have a role in their regulation in tumors. For instance, recent studies shows that Nrp-1 induces α5β1 integrin-mediated fibronectin fibril assembly, involved in regulation of matrix stiffness and tumor progression [100]. In addition Nrp-1, acting as TGF-β1 co-receptor, regulates the phosphorylation of Smad proteins and activate the fibroblasts promoting their conversion in myofibroblasts [101], described to support tumor growth and dissemination. Notably, it has been reported how leukocytes and CAFs can regulate each other and, together, contribute to cancer progression [83, 84]. In fact it has been shown that CAFs can regulate the activation of several immune cells and, by producing pro-inflammatory molecules, can recruit BMDCs able to promote tumor angiogenesis [102].

These data suggest therefore that Sema3s, their receptors and maybe other Semas may regulate the cross-talk between CAFs and inflammatory cells, and together, may contribute to the fine regulation of cancer microenvironment and tumor progression.

Concluding remarks

Work over the past decade has revealed increasingly important roles of semaphorins in vascular development and cancer, and has led to the identification of many new interactions between specific semaphorins and their receptors (Fig.1.). These unique ligand-receptor interactions regulate diverse aspects of vascular development and cancer progression, although we are still in the beginning stage of understanding these biological processes and their dynamic regulation. Many intriguing questions with respect to semaphorin signaling and function remain to be answered. For example, how are the different binding modes between semaphorins and receptor(s) (one ligand with multiple receptors or vice versa, forward or reverse signaling, trans or cis interactions) regulated? How are different ligand-receptor pairs assigned to different aspects of endothelial cell behavior, vessel sprouting and network formation? One important future challenge is to understand precisely how semaphorin ligand binding translates into plexin activation and downstream signaling event in vivo. Semas and their receptors are abnormally expressed during tumor progression both in cancer and stroma cells and their deregulated signal pathways my support or inhibit tumor growth and

angiogenesis. A growing body of evidences demonstrate that these versatile roles of Semas in tumors depend on the different Plexins and Nrps complexes present in different tumor types and on their interaction with receptor tyrosine kinases and integrins. For instance, current studies shows that the cleaved 61 kDa form of Sema3E, binding PlexinD1, induces the association to the ErbB2 receptor in tumor cells, [75] and that Sema4D binding Plexin-B1, induces Met activation [26] promoting, in both cases, metastatic spreading. In addition, recent data describes that Nrp-1 forms a complex with EGFR controlling its activation in tumors [103]. These receptors complexes are differently expressed in tumor and stroma cells (*i.e*. ECs, pericytes, BMDCs and CAF) and can activate multiple signaling modules that can impair or promote cancer growth, angiogenesis, and metastasis dissemination. A better understanding of the global signaling partners and of the cross-talk between the multiple Semas signaling in the different cancer cell types will allow to better predict the outcome of the anti-angiogenic and anti-tumor therapies and to improve their efficacy.

Semaphorins

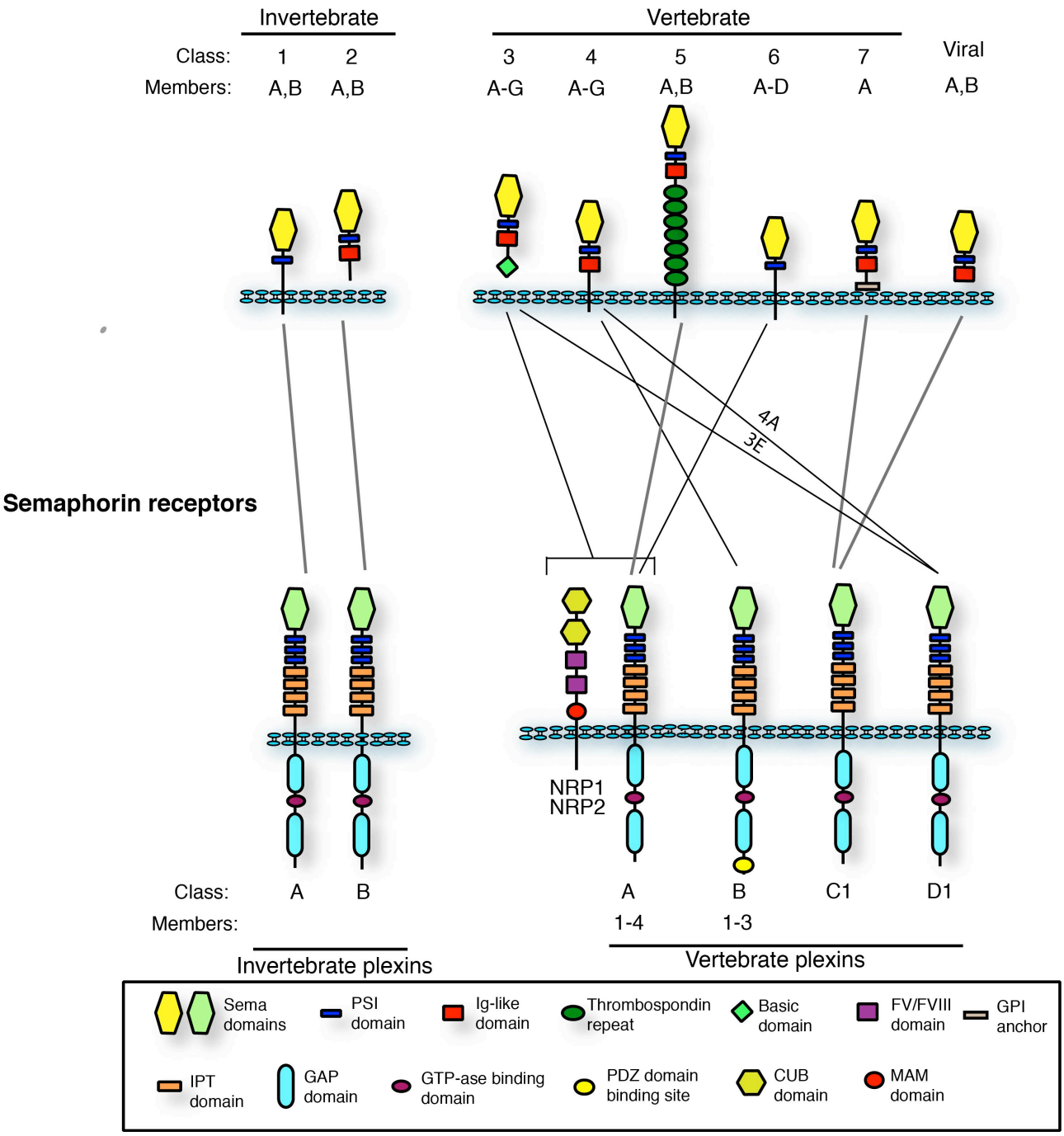

Fig.1.Schematic representation of specific interactions between semaphorins and their receptors (neuropilins and plexins). Semaphorins can be categorized into eight distinct classes. Class 1 and 2 semaphorins are found in invertebrates, whereas classes 3-7 are expressed in vertebrates. The last class of semaphorins includes those encoded by viruses. Class 2 and 3 semaphorins as well as viral semaphorins are secreted. Class 4, 5 and 6 semaphorins are transmembrane proteins and those in class 7 are membrane-anchored via glycophosphatidylinositol (GPI). All semaphorins consist of a large Sema domain and PSI domain. Additional domains that are present in semaphorins include immunoglobulin (Ig)- like domains and thrombospondin repeats. Semaphorins signal through plexin receptors. Plexin A and Plexin B are found in invertebrates. In contrast, vertebrates have four A-type plexins, three B-type plexins, one C-type plexins and one D-type plexin. Plexins contain a Sema domain as well as PSI and IPT domains. Moreover, the cytoplasmic domain of plexins contains two GAP domains, a GTP-ase binding domain and a PDZ domain (characteristic only for B-type plexins). In invertebrates, Sema1s and Sema2s signal through PlexinA and PlexinB, respectively. In vertebrates, Sema3s, Sema5s and Sema6s signal through PlexinAs, with Sema3s also requiring neuropilins (Nrp1 or Nrp2) to facilitate signaling. Neuropilins are transmembrane receptors that are comprised of two complement-like (CUB) domains, two FV/FVIII coagulation factor-like domains and a meprin-like MAM domain and short cytoplasmic tail. Sema3E can also bind directly to PlexinD1 without neuropilins. Class 4 semaphorins interact with PlexinBs while Sema4A can bind to PlexinD1. Sema3A has been suggested to bind to Nrp1/PlexinD1 complex. SEMA7A and viral semaphorins interact with PlexinC1. In addition, Semas and their receptors can also modulate multiple signals in tumors by interacting with receptor tyrosine kinases and integrins (not shown here due to the limitation of space). Semas, Nrps and Plexins are aberrantly expressed in human cancer and tumor microenvironment cells. Different Semas and their receptors, can be differently over-expressed or down-modulated or mutated, depending on the tumor types. Different Semaphorins and their respective receptor(s) regulated different types and aspect of developmental angiogenesis and tumor progression.

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